Conformational Analysis of HIV-1 Protease Inhibitors: 2. Thioproline P_1' Residue in the Potent Inhibitor KNI-272

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Received 6 August 1996; accepted 21 November 1996

ABSTRACT: The very potent HIV-1 protease (HIV-PR) inhibitor, KNI-272, contains a norstatine–thioproline linkage at P_1–P_1'. The three-dimensional crystal structure of this compound bound to HIV-PR has recently been determined [Baldwin et al., *Structure*, **3**, 581 (1995)]. The crystal structure reveals a number of interesting interactions previously unseen in bound HIV-PR inhibitors. Here, we employ high-level *ab initio* calculations and molecular modeling to ascertain the strain energy of the bound conformation of the norstatine–thioproline portion of KNI-272. Baldwin et al. suggested that two of the reasons for the high potency of KNI-272 are the rigidity of its backbone and a strong preference for the norstatine–thioproline amide linkage to adopt a trans conformation. Our analysis shows that, on the contrary, there is still considerable flexibility in the backbone of the norstatine-based inhibitor. Furthermore, in the gas phase and in solution, there are both cis and trans conformations of the norstatine–thioproline amide linkage which are low in energy. However, when bound in the active site of HIV-PR, KNI-272 clearly has a strong preference for a trans conformation, which enables the formation of hydrogen bonds to the flap water. Our calculations, at level up to MP2/6-31++G**//HF/6-31G*, suggest that the bound, trans amide conformation of the norstatine–thioproline ''core'' is still strained by 2–3 kcal/mol, primarily due to the placement of the P_1' thioproline carboxamide. This result is consistent with those previously obtained for the related protease inhibitor Ro 31-8959 (Saquinovir), which also requires a carboxamide to adopt a high-energy rotamer to preserve a good hydrogen bond to the flap water. However, the strain of the bound conformation of KNI-272 is clearly lower than that of Saquinovir. In addition, because the norstatine linkage

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This article includes Supplementary material available from the authors upon request or via the Internet at ftp.wiley.com/public/journals/jcc/suppmat/18/1151 or http://journals.wiley.com/jcc/

CCC 0192-8651 / 97 / 091151-16

does not contain a basic amine (as do Saquinovir and JG-365, for example) it should be easier to desolvate, which also assists in binding. The relationship between KNI-272, JG-365, Saquinovir, and P_1' proline-containing substrate also is discussed. © 1997 by John Wiley & Sons, Inc. *J Comput Chem* **18**: 1151–1166, 1997

Keywords: conformational analysis; HIV protease inhibitors; thioproline; KNI-272; Saquinovir; JG-365

Introduction

KNI-272 (**1**, Fig. 1) is an extremely potent inhibitor of HIV-1 protease (HIV-PR) which contains an allophenylnorstatine–thioproline linkage at P_1–P_1'.[1–5] It is currently in phase I clinical trials.[6] In addition to its obvious therapeutic value, the high potency of this compound makes it a good candidate for structural and modeling studies aimed at improving our understanding of the binding of HIV-PR inhibitors. KNI-272 also is reminiscent of other potent HIV-PR inhibitors which contain a five- or six-membered ring at P_1'. These include JG-365[7,8] (**2**, Fig. 1) and Ro 31-895,[9,10] also known as Saquinovir (**3**, Fig. 1). The latter compound was recently approved for use in the treatment of AIDS patients in the United States.

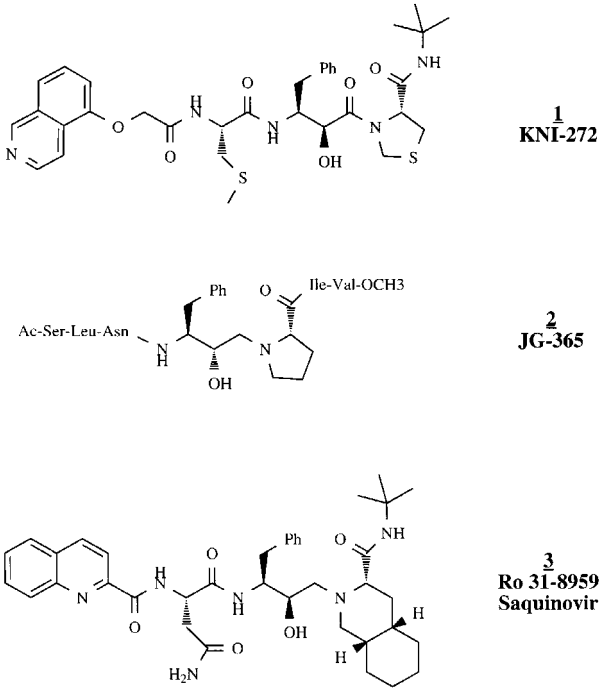


FIGURE 1. Two-dimensional structures of the HIV-PR inhibitors mentioned in this work: KNI-272 (**1**); JG-365 (**2**); and Ro-31-8959 (**3**).

Compounds such as **1**–**3** are of great interest for several reasons. First, they are all quite potent inhibitors of HIV-PR. Second, one is now an approved drug, and another is currently undergoing human clinical trials. Third, the structure–activity relationships for compounds in these classes is quite complex. Changing the stereochemistry of the central hydroxyl group, or changing the P_1' ring, can lead to large changes in binding potency.[11] Fourth, compounds such as **1**–**3** are interesting because, conceptually, they are reminiscent of the Phe–Pro dipeptide which occupies the P_1–P_1' position in several of the naturally occurring substrates for HIV-PR. One may speculate on the structural relationship between the P_1' substrate and the P_1' ring substituent in these inhibitors.

Recently, the three-dimensional crystal structure of KNI-272 bound in HIV-PR has been determined.[12] The crystal structure indicated a bound water molecule bridging between the inhibitor and one of the two catalytic aspartate residues. In addition, the backbone carbonyl of the inhibitor norstatine P_1 residue is within hydrogen bonding distance of the other catalytic aspartate.[12]

Given the novelty and high potency of KNI-272, as well as its therapeutic relevance, we thought it worthwhile to examine in detail the conformational preferences of this molecule. In this article, we describe a series of *ab initio* calculations on prototype compounds **4**, **5**, and **6** (Fig. 2) which contain the essential portions of the norstatine–thioproline linkage. A similar approach has been used[13,14] to study and help explain the conformational preferences in Saquinovir (**2**). We have also modeled KNI-272 in various possible bound conformations within the active site of HIV-PR to help rationalize the reported crystal structure. We have modeled the bound conformation of the diastereomeric molecule, epi-KNI-272, which has an *R* stereochemistry at the central hydroxyl group, and is known to be considerably less potent. Finally, we have modeled P_1' proline-containing substrates

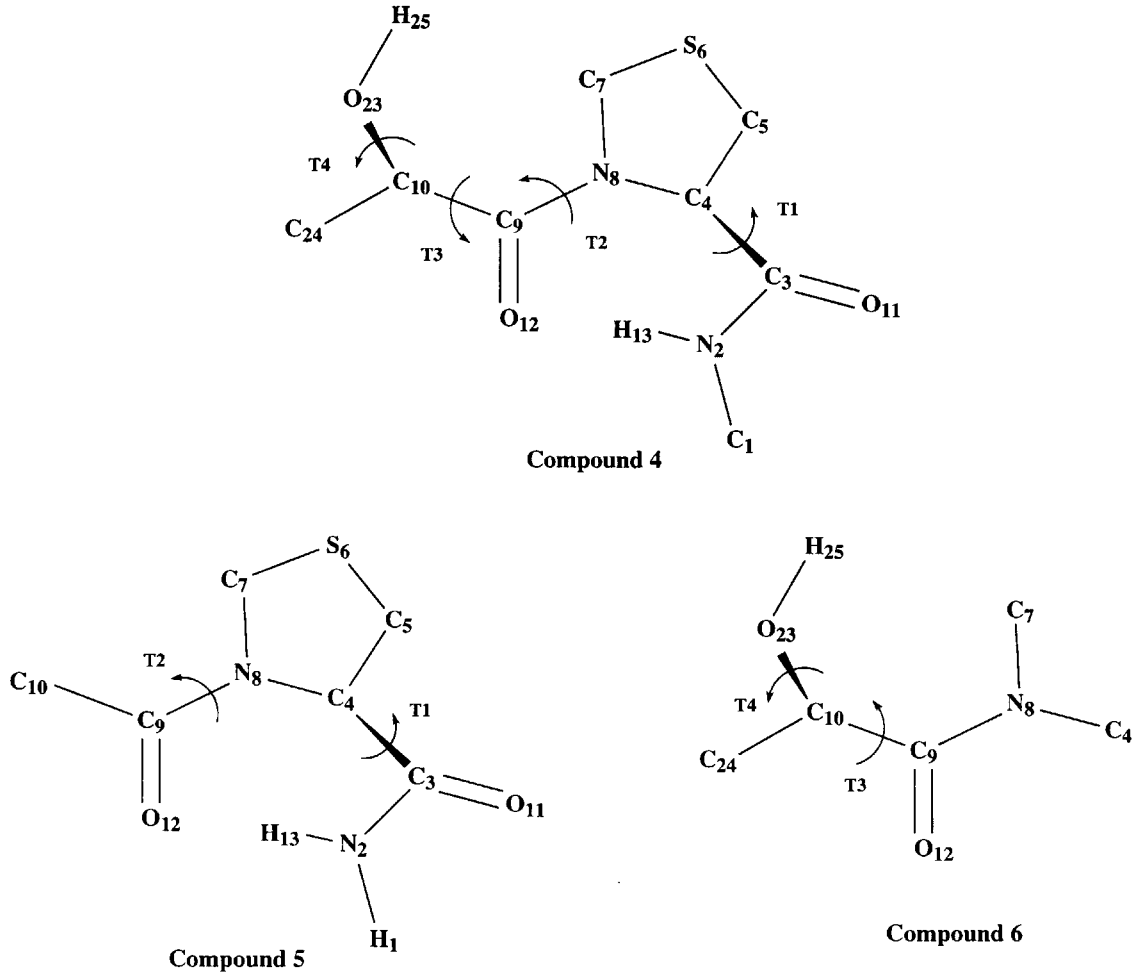


FIGURE 2. Two-dimensional structures of the prototype compounds (**4**–**6**) used in this study.

and compared them to all available crystal structures and models for P_1' ring-containing inhibitors.

Computational Details

Molecule **4** (Fig. 2) contains the central portion (P_1–P_1') of KNI-272 (**1**). There are four torsions of interest in molecule **4** which can adopt distinct conformations: T1, N2—C3—C4—C5; T2, C4—N8—C9—C10; T3, N8—C9—C10—O23; and T4, C9—C10—O23—H25. There are three, two, three, and three local minima, respectively, for these four torsions. T2 is the amide bond, which can be either cis or trans; the other three torsions can each adopt trans, gauche$^+$, or gauche$^-$ conformations. Thus, one might expect that, in principle, there are $3 \times 2 \times 3 \times 3 = 54$ reasonable starting conformations for detailed energy calculations. The conformational analysis is further complicated by two additional factors. First, the thioproline is flexible, and may adopt several conformations (Fig. 3). Second, the amido group may adopt a pseudoaxial or pseudoequatorial orientation with respect to the five-membered ring. (The pseudoaxial conformers are expected to be lower in energy, but a few pseudoequatorial conformers may be expected to have reasonable energies, and cannot be discounted *a*

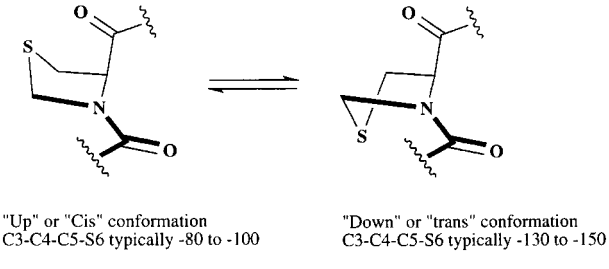


FIGURE 3. Two possible conformations (pseudorotamers) of the thioproline ring.

priori.) The barriers to interconversion in five-membered ring systems are, in general, quite small,[15] and so one would not expect that for each of the 54 rotational conformers there will be multiple ring conformations and distinct pseudoequatorial and pseudoaxial local minima. However, some number of additional conformations is to be expected. Considering the size of compound **4** and the complexity of *ab initio* calculations, the large number of conformations is troublesome.

To simplify our analysis, we decided to subdivide the problem. We first carried out a series of calculations on molecule **5**. Based on our previous work,[13,14] we anticipated that a detailed energy profile for torsion angle T1 would be critical to help us understand the strain energy in the bound state of KNI-272 (**1**). We rotated T1 in 30° increments and allowed the norstatine amide bond, T2, to freely adopt a cis or trans orientation. For each combination of T1 and T2, we generated two starting conformations of the thioproline ring, one with the sulfur tipped up, on the same side of the ring as the carboxamide, and one with the sulfur tipped down, away from the carboxamide (Fig. 3). Thus, a total of 48 distinct conformations of molecule **5** ($12 \times 2 \times 2$) were studied. Each conformation was optimized using the HF/3-21G* and then the HF/6-31G* basis sets using GAUSSIAN 94.[16] All internal coordinates except for T1 were free. In several of the 48 conformations, the C3—C4—C5—S6 torsion had to be held fixed to prevent pseudorotation of the thioproline ring. Supplementary Table S1 contains the complete set of results. The relative energies are plotted in Figure 4.

There are four ''categories'' of conformations: the amide may be cis or trans, and the sulfur may be tipped up or down. For each category, we took the lowest energy conformer from Table S1 and reoptimized at the HF/6-31G* level allowing the T1 torsion angle to relax. We then carried out MP2/6-31G* single-point determinations on these relaxed geometries. The results are given in Table I.

Next, we turned to molecule **6**, which contains the central hydroxy group as well as the statine carbonyl and a portion of the thioproline ring. We investigated the conformational preferences around bonds T3 and T4. Each of these torsions is likely to

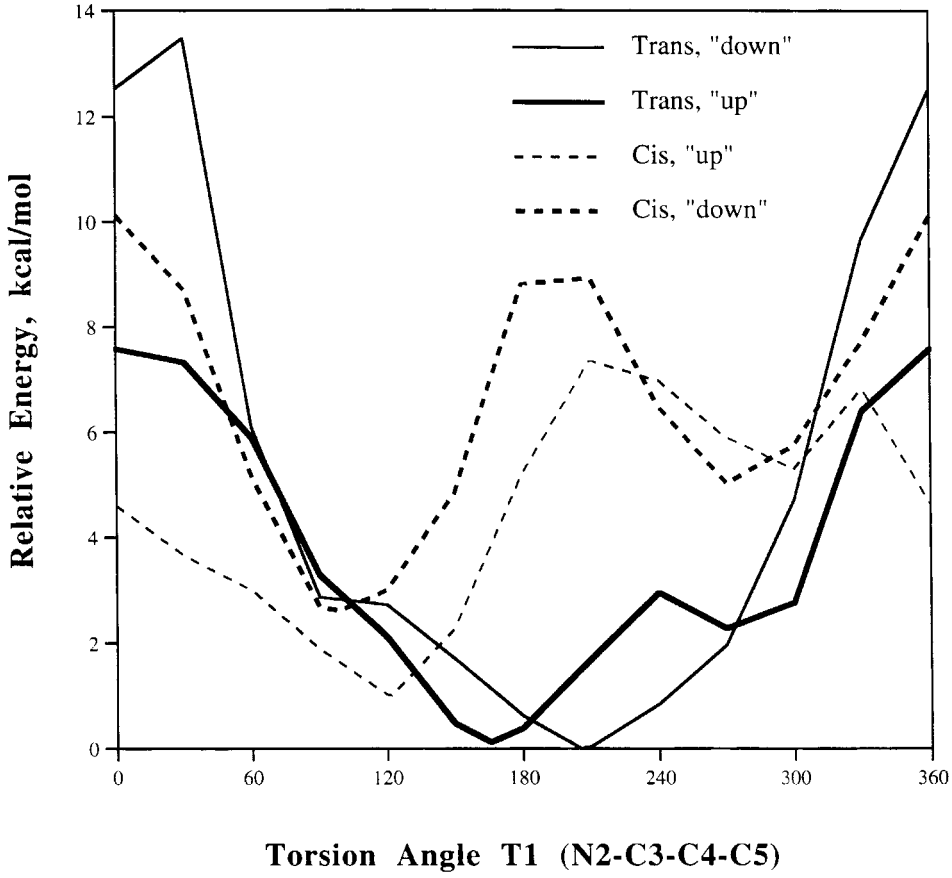


FIGURE 4. Relative energies of cis and trans amide conformers of compound **5**.

have three local minima (trans, gauche$^+$, and gauche$^-$). Accordingly, we carried out calculations on molecule **6** starting from each of the possible low-energy conformations. The results are summarized in Table II.

Finally, we investigated compound **4**. Using the information in Table I and II, we selected ~50 distinct starting conformations that combined the best features of molecules **5** and **6**. We also ensured that all conformations possessing an intramolecular hydrogen bond were included. Finally, we also included conformations which CHARMM[17,18] suggested could have reasonable relative energies. In all, ~60 conformations were studied. Each compound was energy minimized at the STO-3G* level holding the values of T1–T4 fixed. Next, a 3-21G* optimization with T1–T4 again fixed was carried out. This was followed by a HF/6-31G* optimization in which all torsions were allowed to relax. If the conformation changed dramatically, that is, if a different local minimum was found, we would reoptimize at the HF/6-31G* level with the T1–T4 torsions fixed, and then relax all torsions in a subsequent optimization. The results are given in Supplementary Table S2. In several cases, different starting conformations converged to the same structure. Also, some of the optimized conformations had relative energies greater than 10 kcal/mol above the global minimum. After removal of the duplicate and high-energy conformations there are 32 distinct conformations which remain. These are named A1 through A32 in Supplementary Table S2.

We also used the X-ray data for KNI-272[12] to generate the analogous conformation of molecule

TABLE I.
Fully Relaxed Local Minima Conformations of Compound 5 Compared to Bound Conformation from X-Ray Crystallography.

Family[a]	N2—C3—C4—C5	C3—C4—C5—S6	C4—C5—S6—C7	C5—S6—C7—N8	S6—C7—N8—C4	C7—N8—C4—C5	C3—C4—N8—C9	C4—N8—C9—C10	HF/6-31G*	MP2/6-31G*
Trans, "down"	−154	−136	31	−39	37	−13	−87	−171	0.00	0.00
Trans, "up"	165	−87	−35	23	−3	−22	−86	−173	0.11	0.72
Cis, "up"	121	−87	−38	27	−9	−18	−90	7	1.00	1.96
Cis, "down"	98	−153	37	−37	27	0	−81	10	2.60	3.24

[a] Family defines the value of T2 and the conformation of the five-membered ring. Cis and trans refer to the amide bond (T2). "Down" and "up" refer to the tip of the thioproline sulfur atom either away from ("down") or toward ("up") the carboxamide.

TABLE II.
Summary of Results for Calculations on N,N-Dimethyl-2-Hydroxy-Propionamide[a] (Compound 6)

CCCN	OCCN (T3)	HOCC (T4)[b]	Energy[c]	Rel. E[c]
184	62	173	−399.91102	3.97
196	72	67	−399.91289	2.79
184	65	−60 (f)	−399.90732	6.29
78	198	−21	−399.91734	0.00
63	181	30 (f)	−399.91380	2.22
90	211	183	−399.90410	8.31

[a] Another common name for this compound is N,N-dimethyl-lactamide.

[b] (f) indicates that a torsion angle was constrained during the optimization.

[c] Absolute energies in hartrees, relative energies in kilocalories per mole.

4, called X2. Conformation X1 had all torsion angles frozen at their crystallographic positions except for the HOCC (T4) angle, which was allowed to relax. Conformation X3 allowed all torsion angles to relax except for T1, whereas conformation X4 had all torsions relaxed. The data for X1–X4 also are given in Supplementary Table S2. For convenience, the compounds in Supplementary Table S2 are ranked by relative energy. Furthermore, each conformation has been classified based on the values of torsion angles T1 through T4. For example, if all four torsions are trans, the conformation would be categorized as TTTT. Finally, for each conformation the intramolecular hydrogen bonds are listed.

In Table III, we summarize the relative energies of several of the low-energy conformations of molecule 4 from the *ab initio* calculations, as well those torsions seen in the binary crystal structure of 1.[12] In addition, MP2 single point calculations at the 6-31G* and 6-31++G** levels were carried out on the five lowest energy conformations (A1 through A5) as well as the X-ray conformation. These data are also given in Table III.

Finally, we started from our global minimum structure, A1, and rotated the T1 torsion in 30° increments to better understand the energetics of this bond rotation. All other internal coordinates were allowed to fully relax. These optimizations were again carried out at the HF/6-31G* level. Results are given in Table IV. For convenience, we also include the data for conformations A1 and X4, the two local minima along this potential surface. A plot of relative energy versus T1 torsion angle is given in Figure 5. For comparison, the conformational data for two relevant thioproline structures from the Cambridge Structural Database[19] also are given in Table IV, and these two molecules are illustrated in Figure 6.

TABLE III.
Low-Energy Conformations of Compound 4 Compared to Bound Conformation from X-Ray Crystallography.[a]

Structure	T1–T2–T3–T4 category	T1 2–3–4–5	3–4–5–6	4–5–6–7	5–6–7–8	6–7–8–4	7–8–4–5	T2 7–8–9–10	T3 8–9–10–23	8–9–10–24	T4 9–10–23–25	HF/6-31G* rel. energy	MP2/6-31G* rel. energy	MP2/6-31++G** rel. energy
A1	TTTG	171	−87	−38	27	−10	−17	−9	151	−86	32	0.00	0.00	0.23
A2	TCTG	122	−86	−37	25	−6	−21	172	149	−89	33	0.31	0.78	0.00
A3	TTTG	129	−87	−38	28	−9	−18	−14	155	−82	28	1.15	1.93	N/A
A4	GTGG	−151	−140	34	−39	34	−9	−13	73	−161	68	1.46	1.09	N/A
A5	TTgg	−149	−141	35	−39	35	−9	−15	−30	93	−68	1.84	1.80	N/A
X3	gTTG	−92	−93	−9	−9	29	−37	11	152	−90	27	2.30	4.14	N/A
X4	gTTG	−76	−85	−19	0	23	−39	8	149	−89	32	1.61	3.55	3.05

[a] Angles in degrees, relative energies in kilocalories per mole. All geometries are HF/6-6-31G*. The energy of the global minimum is −1042.11256, −1044.11926, and −1044.28510, respectively. See text and Figure 2 for definitions of T1–T4.

Results

COMPOUND 5

The data in Table I and Supplementary Table S1, along with the plot of relative energy versus torsion angle in Figure 4, allow us to deduce certain trends in the conformational preferences of this molecule. These can be summarized as follows:

(a) Whenever the T2 torsion (the amide) is trans, the preferred value of T1 (the N2—C3—C4

TABLE IV.

Thioproline Conformations for Compound 4. Comparison of Ab Initio Results for T1 Rotation with KNI-272 Bound X-Ray Structure and Small-Molecule Thioproline X-Ray Data.

Structure	T1 2–3–4–5	3–4–5–6	4–5–6–7	5–6–7–8	6–7–8–4	7–8–4–5	T2 7–8–9–10	T3 8–9–10–23	8–9–10–24	T4 9–10–23–25	HF/6-31G* rel. energy
R1	0	−83	−17	−7	33	−47	−13	164	−73	20	6.47
R2	29	−88	−14	−9	33	−44	−16	167	−71	18	6.39
R3	59	−97	−35	34	−25	0	−15	156	−82	27	5.01
R4	90	−97	−36	34	−24	−1	−14	156	−82	27	2.33
R5	120	−89	−38	30	−13	−14	−15	156	−82	27	1.26
R6	150	−86	−39	28	−10	−18	−5	150	−88	32	0.65
A1	171	−87	−38	27	−10	−17	−9	151	−86	31	0.00
R7	180	−88	−37	27	−10	−16	−10	152	−86	31	0.11
R8	210	−92	−32	25	−11	−12	−12	153	−85	31	1.49
R9	240	−94	−30	24	−12	−9	−7	151	−86	32	2.94
R10	270	−93	−10	−9	29	−38	10	151	−86	29	1.87
X4	284	−85	−19	0	23	−39	8	149	−89	32	1.61
R11	300	−83	−20	−1	26	−42	5	150	−87	30	2.10
R12	330	−83	−18	−6	32	−48	−1	157	−81	25	5.32
BEHPEJ	131	−81	−36	17	6	−34	180	N/A	N/A	N/A	N/A
DIWNAY	129	−82	−30	10	14	−37	0	N/A	N/A	N/A	N/A

[a] Angles in degrees, energies in kilocalories per mole. *Ab initio* geometries are HF/6-31G*. The absolute energy of A1 is −1042.11256. The structures from the CSD are given in Figure 6. For these molecules, T3 and T4 are not present (N/A).

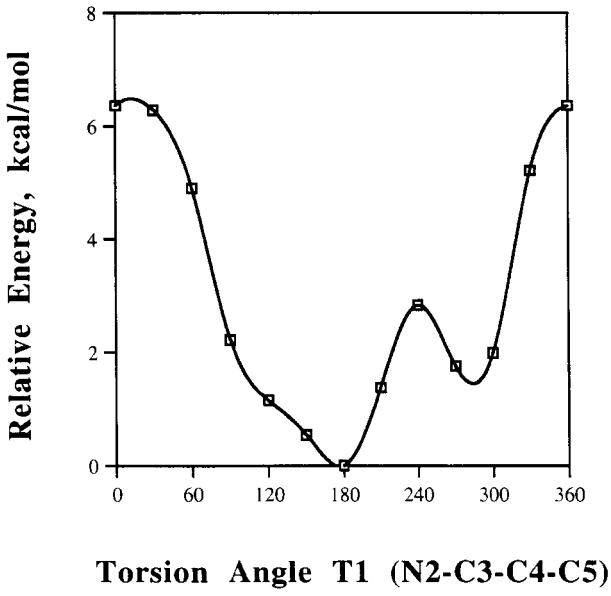


FIGURE 5. Several low-energy conformations of compound **4** from *ab initio* calculations, the KNI-272 bound X-ray structure, and small-molecule x-ray data.

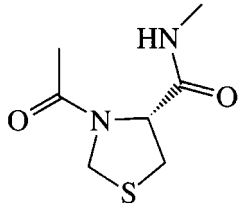
—C5 torsion) is approximately 180°; when the T2 torsion is cis, the preferred value of T1 is around 120°.

(b) At the HF/6-31G* level there is a small preference (~ 1 kcal/mol) for a trans amide rather than a cis amide. At the MP2/6-31G*//HF/6-31G* level, this preference increases to ~ 2 kcal/mol (Table II).

(c) The potential energy surface of the trans amide conformations (Fig. 4) tend to be more shallow than for the cis amide conformations. For example, 14 of 24 trans amide conformations are within 3 kcal/mol of the lowest energy trans amide conformation, whereas only 7 of 24 cis amide conformations are within 3 kcal/mol of the lowest energy cis amide conformation.

(d) There is not much of a preference, in general, for any particular conformation of the thioproline ring. Both the sulfur tipped up (cis to the carboxamide) and tipped down are similar in energy (Fig. 4). Not surprisingly, other ring torsions (Table I) are strongly correlated with the choice of sulfur orientation.

BEHPEJ



DIWNAY

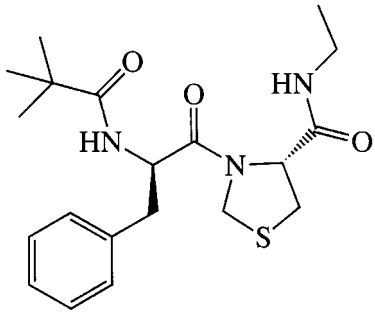


FIGURE 6. Molecules found in the CSD that contain thioproline.

COMPOUND 6

T3 and T4 are the two torsion angles being varied in this molecule (Fig. 2). The results in Table II show, not surprisingly, that conformations which allow the formation of an intramolecular hydrogen bond between the hydroxyl proton and the carbonyl oxygen are preferred. In the global minimum conformation, the T4 torsion (H—O—C—C) is nearly eclipsed (~ 21°) to form this hydrogen bond. Other conformations that do not form an intramolecular hydrogen bond are possible, with energies from 2 to 4 kcal/mol higher. These conformations of torsions T3 and T4 will need to be considered for molecule **4**, because other intramolecular hydrogen bonds will be possible in this more complex molecule.

COMPOUND 4

The data for compound **4** are given in Tables II and IV and Supplementary Table S2. Clearly both cis and trans amides (T2) are found. In addition, the thioproline ring conformation is found in both the ''up'' and ''down'' conformations (as measured by the C3—C4—C5—S6 torsion angle). Finally, there is considerable flexibility in the conformation of the thioproline ring. Together, these variations produce a large number of low-energy conformations. There are 17 conformations within 4 kcal/mol of the global minimum (A1–A16 plus the X-ray conformation).

The global minimum structure, A1, is of family TTTG. This is identical to the X-ray conformation except for torsion T1, which is gauche$^-$ in the X-ray structure (conformations X3 and X4 in Table III, family classification = gTTG). The relative energy of the X-ray conformation, X2, is around 4 kcal/mol. However, five-membered rings are quite flexible, and a more realistic measure of the strain energy of the X-ray conformation is obtained by relaxing the ring conformation. Thus, conformation X3, which only fixes the T1 torsion (N2—C3—C4—C5), has a strain energy of 2.3 kcal/mol at the HF/6-31G* level, and 4.1 kcal/mol at the MP2/6-31G* level. Furthermore, conformation X4, which has no torsions fixed, moves T1 by 16°, from $-92°$ to $-76°$, and has a relative energy of 1.61 kcal/mol at the HF/6-31G* level, 3.55 kcal/mol at the MP2/6-31G* level, and 2.82 at the MP2/6-31++G** level. When conformations X3 and X4 are compared with the global minimum, A1, it can be seen (Table III) that the largest difference is in the T1 torsion, which is trans in the global minimum, but gauche$^-$ in the X-ray conformation. In addition, the conformation of the thioproline ring is somewhat different.

Because of the difference in thioproline ring conformation between the global minimum, A1, and the related X-ray structure, X4, and to better understand the conformational preferences around the critical T1 angle, we carefully studied the change in energy as a function of the rotation of T1. The data in Table IV (plotted in Fig. 5) show that for molecule **4**, as for molecule **5**, there are clearly preferred values for T1. There is a 1.6-kcal/mol preference for T1 to be trans rather than gauche$^-$; there is no local minimum at gauche$^+$. As T1 rotates, the ring changes conformation; several of the ring torsions can deviate by as much as 30°.

It is of interest to compare the results of the *ab initio* calculations with the two thioproline-containing molecules in the Cambridge Structural Database.[19] These two compounds, BEHPEJ[20] and DIWNAY,[21] shown in Figure 6, both have T1 values of $\sim 130°$. According to our calculations on molecule **4** (Fig. 5), this T1 value is relatively strain-free (approximately 1 kcal/mol) when the amide torsion (T2) is trans. However, BEHPEJ exists in a cis amide conformation. To estimate the strain in this molecule we must consider the data from molecule **5**. The graph in Figure 4 (the ''cis, up'' curve) shows that this torsion is again only ~ 1 kcal/mol stained at the HF/6-31G* level. The conformation of the thioproline ring of DIWNAY is, in general, in reasonable agreement with the *ab initio* data for X4, the fully relaxed conformation derived from the bound X-ray structure of KNI-272.

Discussion

CONFORMATIONAL ANALYSIS OF COMPOUNDS 4–6

As described in the Results section, our analysis of compounds **4**–**6** indicate that there are, as expected, many low-energy conformations possible for compounds such as KNI-272 (**1**). An important observation is that both cis and trans amide conformers (torsion T2) are found to be low in energy. The most important variables in the gas-phase calculations are: (a) the ability to form at least one intramolecular hydrogen bond; and (b) the value of the T1 torsion angle (N2—C3—C4—C5), which determines the relationship of the thioproline ring and the carboxamide group. Neither of these variables implies any preference for a trans amide bond; both cis and trans are possible, and we find many low-energy conformations with both cis and trans amide configurations.

In solution, or in the active site, KNI-272 may form intermolecular hydrogen bonds with water or HIV-PR, thus stabilizing certain conformations which, according to the *ab initio* calculations, are high in energy. Intramolecular hydrogen bonds between the backbone carbonyl of the residue preceding proline and the amide group of the residue following proline (a γ-turn) are known in cyclic peptides.[22] However, the formation of a β-turn involving proline is much more commonly found[23,24] and is generally through to be energetically preferable.[25] This suggests that if there are opportunities for the norstatine–thioproline portion of KNI-272 to form intermolecular hydrogen bonds to HIV-PR, those hydrogen bonds will probably be preferred. In any event, it is obvious that stable, bound conformation should form several hydrogen bonds, regardless of whether they are intra- or intermolecular.

However, the data clearly show that certain rotamers of T1 are energetically preferable, even after accounting for hydrogen bonding. In other words, the T1 torsion angle, like most torsions, has certain intrinsic preferences. The analogous ψ angle of proline is likewise known to have definite preferences for similar values.[26,27] This is independent of the nature of the hydrogen bonding involving the proline carbonyl group and the amide

which follows it. This is essentially the same result that we determined in our analysis of the bound state and conformational preferences of Saquinovir (**2**).[13,14] We can thus see the *ab initio* results to help determine the strain energy (*vide infra*).

Finally, it is worth mentioning that dielectric effects are not expected to play a large role on these relative energies. In our earlier study on Saquinovir,[13,14] we found that the conformational preferences around the P_1' DIQ moiety were hardly affected by dielectric, even though it is a formally charged system. So, for the neutral norstatine system, we anticipate that dielectric effects will play only a small role.

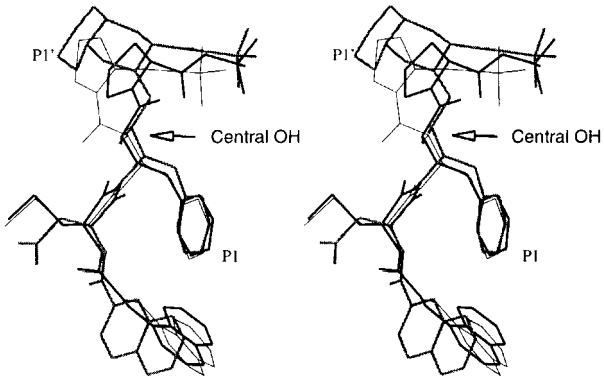


FIGURE 7. Comparison of the bound X-ray conformations of KNI-272 (thick, dark lines), Saquinovir (thick, gray lines), with the modeled conformation of epi-KNI-272 (thin, dark lines).

BOUND CONFORMATION AND STRAIN ENERGY OF KNI-272

The crystal structure of KNI-272 was determined and carefully examined by Baldwin et al.[12] They showed that, in addition to strong interactions with the flap water and the catalytic aspartates, there are several interesting waters mediating between the enzyme and inhibitor. The norstatine–thioproline amide bond is trans. Furthermore, there is a gauche relationship between the P_1 side chain and the central hydroxyl group, as is found in all classes of HIV-PR inhibitors (discussed at greater length in the Discussion section).

The bound conformation of KNI-272 seen by Baldwin et al.[12] still as considerable strain in the norstatine–thioproline region. We may estimate this strain energy either by using the data for molecule **5** (Table I and Supplementary Table S1, Fig. 4) or molecule **4** (Table III and Supplementary Table S2, Fig. 5). Our estimate from molecule **5** is ~ 3 kcal/mol in the gas phase at the MP2/6-31G*//HF/6-31G* level. The majority of this strain is due to a nonoptimized, high-energy carboxamide rotamer (torsion angle T1, N2—C3—C4—C5) which is required to preserve the flap water hydrogen bond. Using molecule **4** (Table III), we show that the relative energy of the X-ray conformation, X3 (which only freezes the T1 torsion angle), is 2.3 kcal/mol above the global minimum, A1. This estimate allows for geometrically subtle but energetically important changes in the thioproline ring conformation, as discussed earlier in the Results section. The completely relaxed X4 conformation has a relative energy of ~ 1.6 kcal/mol at the HF/6-31G* level. At the MP2/6-31++G** level, this relative energy rises to ~ 2.8 kcal/mol. So, both molecules **4** and **5** give similar estimates of the strain energy of the bound conformation of the ''core'' of KNI-272.

Our data further shows that the relative energy of prototype molecule **5** (Table I) changes dramatically with T1. Similarly, large changes in relative energy are seen in molecule **4** when T1 is changed. For example, changing the T1 torsion from trans to gauche$^-$ (conformation A1 versus X3, Table III) increases the energy by more than 2 kcal/mol. Even more dramatically, changing the T1 torsion by only 16° from −92° to −76° (conformation X3 versus X4) leads to a stabilization of 0.7 kcal/mol at the HF/6-31G* level, and 0.6 kcal/mol at the MP2/6-31G* level. The critical nature of the T1 torsion angle is qualitatively the same as that previously obtained[13,14] for the Roche drug, Saquinovir (**3**). This is not surprising in light of the obvious similarities between molecules **1** and **3** in the P_1' region (Fig. 7). The bound conformation of Saquinovir is somewhat more strained (~ 5 kcal/mol) than that of KNI-272 (~ 2–3 kcal/mol) because of the fused cyclohexyl of the DIQ bicyclic ring system, which introduces additional steric repulsions.[13,14] In this sense, KNI-272 can be considered a somewhat improved compound because the total strain energy of the bound state is reduced. Our best estimate is that the strain energy has been reduced by 2–3 kcal/mol.

Five-membered rings are known to pseudorotate freely,[15] and it is sometimes difficult to precisely determine the conformation of such rings. For this reason it is of interest to compare the conformation of the thioproline ring in the X-ray structure of KNI-272 versus the *ab initio* models, as well as other crystal structures from the Cambridge Structural Database. This comparison is

made in Table IV. Interestingly, both the experimental data and the *ab initio* calculations show that torsions S6—C7—N8—C4 and C7—N8—C4—C5 are rather different than that found in the KNI-272 structure. It is not clear whether this represents averaging in the bound conformation of the KNI-272 X-ray structure, or a nonoptimal bound conformation. Unfortunately, there is, in general, no simple way to tell from bond lengths, bond angles, or B-factors whether a set of X-ray coordinates represents averaging. In any event, as we have stated, five-membered rings can pseudorotate and adopt a variety of low-energy conformations. In the discussion that follows, we only calculate strain energies by starting from the X-ray structure of the thioproline ring and allowing it to fully relax. This gives us a ''lower limit'' of the strain energy which allows us to deconvolute the other factors leading to strain in the bound conformation, while eliminating the possibility that we are unfairly biasing the analysis by using an artifactual averaged conformation.

Because the strain energy of the bound conformation of KNI-272 is lower than that of Saquinovir and, presumably, some other inhibitors of HIV-PR, one might argue that KNI-272 is ''preorganized.'' Baldwin et al.[12] claim that KNI-272 and the Dupont-Merck cyclic urea inhibitor, XM323,[28] are the only examples of preorganized HIV-PR inhibitors. Our analysis, as described here, shows that KNI-272, although improved relative to Saquinovir, is nonetheless strained to a certain extent. Other work suggests that XM323 likewise suffers from several kilocalories per mole of strain energy (Murcko and Rao, unpublished data). VX-478[29] is representative of another class of HIV-PR inhibitors which is relatively unstrained when bound to the enzyme. Whereas each of these compound classes has *reduced* strain energy in the bound state, none of them is ''strain free.''

Finally, one might argue that KNI-272 has a second advantage relative to Saquinovir: its reduced interaction with hydrophobic residues Val-82 and Ile-84, which are prone to mutation.[30] However, Baldwin et al.[12] showed that the thioproline ring does still make contact with Ile-84, and it has been reported[30] that the I84V mutation reduces potency by roughly 30-fold.

BOUND CONFORMATION AND STRAIN ENERGY OF EPI-KNI-272

It also is of interest to model the bound conformation of epi-KNI-272, which has the reversed stereochemistry (*R*) at the central hydroxyl and has been shown to be 200-fold less potent than KNI-272. Tam and coworkers have likewise shown a 400-fold reduction in potency for closely related norstatine-based inhibitors.[11] Our models suggest that if the P_1-P_1' amide in epi-KNI-272 adopts a cis conformation, it will be able to maintain multiple interactions with catalytic aspartates (Fig. 7). The backbone conformation would then be similar to that found in JG-365[7,8] with a good overlap of the P_1' five-membered rings of the two inhibitors (Fig. 8). To study the strain energy of this portion of epi-KNI-272, Baldwin et al. calculated the conformational preferences of thioproline in the 2-*R*-hydroxyl norstatine linkage by density functional methods.[12] They found the cis conformation to be 8 kcal/mol higher in energy than the trans configuration, although no details were given. This result was judged to be in qualitative agreement with the reduced potency of epi-KNI-272.[12]

We have reexamined this question in light of our models of epi-KNI-272 (Figs. 7 and 8) and our *ab initio* data. Because there is no intrinsic preference for the trans amide conformation, and we must look elsewhere for an explanation of the reduced potency of the epi isomer. For example, it should be possible to demonstrate that the bound conformation of the epi isomer is more strained than that of KNI-272. In our models of epi-KNI-272, the value of the T1 torsion (N2—C3—C4—C5) is

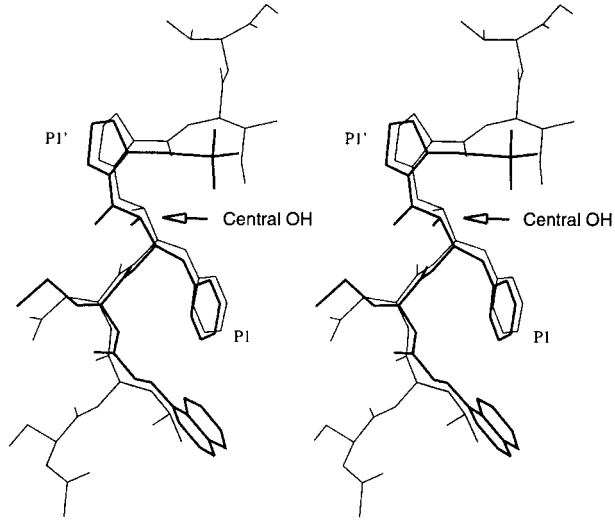


FIGURE 8. Comparison of the modeled conformation of bound epi-KNI (thick lines) with the bound X-ray structure of JG-365 (thin lines).

−115° (versus −89.2° in JG-365). Our calculations on molecule **5** (Table I and Supplementary Table S1, Fig. 4) show that this conformation is ~6 kcal/mol above the global minimum. In the previous section, we showed that our estimate for the strain energy of KNI-272, again using the data in Tables I and S1, and Figure 5, is ~3 kcal/mol. The difference (6 − 3 = 3 kcal/mol) is the ''differential strain'' in the epi diasteomer. This translates into approximately a 100-fold difference in binding, in good agreement with the experimental results on norstatine-based inhibitors from two different labs.[11,12]

Another way to address the loss in potency of the epi isomer is to ask the following question: If the cis conformation is so much higher in energy, why does it not adopt a trans configuration? One reason, of course, is the loss of multiple hydrogen bonds with the catalytic aspartates. In this trans (KNI-272-like) conformation, it is likely to make only one hydrogen bond with the outer oxygen (OD1) of Asp-25′ and no interaction with Asp-25. Furthermore, the hydroxyl is spatially in a trans (*anti*) configuration relative to the P_1 phenylalanine side chain and is very close to the P_1' thioproline ring. Thus, the epi isomer has no effective way to relieve the strain of its norstatine−thioproline moiety: either it adopts a high-energy cis amide conformation, which allows the central hydroxyl to preserve its hydrogen bonds, or it adopts a lower energy trans amide conformation, and loses those hydrogen bonds. This is precisely analogous to the situation of epi-Saquinovir, as previously described.[13,14] In that case, the preferred stereochemistry of the central hydroxyl group is *R*. The *S*-hydroxyl diastereomer is ~200-fold less potent. Our simulations indicated[13,14] that the *S*-diastereomer was forced to choose between preserving the correct placement of the central hydroxyl group, which required adopting a high-energy conformation, or displacing that hydroxyl group and maintaining a low-energy conformation. Either choice would rendered the *S*-diastereomer less potent than the *R*-diastereomer, which could both preserve the hydroxyl group and permit adoption of the low-energy conformation.

It is also important to note that there are two other important differences between KNI-272 and its epi isomer. First, in epi-KNI-272, the P2′ NH group is able to make a direct hydrogen bond to the backbone carbonyl of Gly-27, as is found in JG-365 (Fig. 8). In KNI-272, on the other hand, this NH group makes a hydrogen bond to a water molecule in the P2 pocket. Second, in the epi isomer, the tert−butyl group is not buried as deeply in the P2 pocket, and is making fewer favorable van der Waals interactions. The combination of these two factors will also play a role in the relative affinities of KNI-272 and its epi isomer, although precise quantitation is not feasible.

COMPARISON OF HYDROXYETHYLAMINE AND NORSTATINE

It is of interest to compare the binding modes of molecules **1**–**3**. KNI-272 (**1**) has a core transition state mimic containing hydroxymethyl-carbonyl [norstatine, —CH(OH)C(=O)N—], whereas Ro 31-8959 (Saquinovir, **3**) utilizes a hydroyethylamine [—CH(OH)CH_2N—, also known as HEA] moiety. Both have been shown by X-ray crystallography to have a similar bound conformation: the backbone C(=O)N or CH_2N are in a gauche orientation with respect to the P_1 side chain (Table IV, dihedral #2). This is opposite from that found in all other published HIV-PR inhibitor crystal structures. Tam et al.[11] compared the binding of these two series of compounds and found that a compound with norstatine core (Z—Asn—Phe—[CH(OH)C(=O)N]—Pro—NH—tBu) (IC_{50} = 7 nM) is about 30-fold better than the corresponding hydroxyethylamine inhibitor (IC_{50} = 210 nM). Mimoto et al.[1,2] found the same norstatine compound to be 89 nM in their assay, which makes it only 2.3-fold more potent compared to the hydroxyethylamine analog. In both cases, it is clear that the norstatine analog is more potent than the hydroxyethylamine analog. This is clearly due, in part, to that fact that the bound conformation of the norstatine analog, as discussed earlier, is closer to its global minimum energy conformation. Another factor that may favor the norstatine compounds may be the cost of desolvating the tertiary amine of the HEA-containing inhibitors. This amine is presumably charged in solution, whereas the norstatine is neutral.

As we discussed in the previous section, JG-365 (**2**) likewise incorporates an HEA group. However, the absolute stereochemistry of the central hydroxyl group of JG-365 is opposite to that of KNI-272 (**1**) and, consequently, the backbone conformation (Table IV, dihedral #2) is different.

It also is important to note that all the X-ray structures of HIV-PR complexed with inhibitors from these various classes show only the gauche configuration of the hydroxyl with respect to the P_1 side chain (Table V). Baldwin et al.[12] point out

TABLE V.
Variation of Stereochemistry and Conformational Preferences in Published HIV Protease Binary Crystal Structures.[a]

PDB code	Class	P_1	P_1'	R / S—OH	Dihedral #1[b, e]	Dihedral #2[c, f]
1aaq	HE	Phe	Gly	S—OH	67.8	−170.0
1hps	HE	Phe	Phe	S—OH	84.2	−157.7
5hvp	HE	Leu	Gly	S—OH	119.0	−122.6
8hvp	HE	Leu	Val	S—OH	50.5	−173.9
1hvj	HE	Phe	Phe	S—OH	68.6	−174.8
1hef	HE	Phe	Phe	S—OH	51.8	+174.0
1heg	HE	Phe	Gly	S—OH	60.6	+177.7
1hih	HEA	Phe	N−Chx	S—OH	71.4	−170.1
7hvp	HEA	Phe	Pro	S—OH	50.5	+168.9
(Roche)	HEA	Phe	DIQ	R—OH	51.9	−70.6
1hvp	HEA	Phe	N−Leu	R—OH	46.2	−76.1
1hpx	HMC	Phe	Thio−Pro	S—OH[d]	52.0	−72.1
1hvi	C2-diol	Phe	Phe	R,S-diol	g / g	t / t
1hvk	C2-diol	Phe	Phe	S,S-diol	g / g	t / t
1hvl	C2-diol	Phe	Phe	R,S-diol	g / g	t / t
1hiv	Diol	Chx	Val	R,S-diol	g / g	t / t
1hvr	Cyclic diol	Phe	Phe		g / g	t / t

[a] Abbreviations: HE, hydroxyethylene; HEA, hydroxyethylamine; HMC, hydroxymethylcarbonyl. This group is also known as norstatine.
[b] Dihedral #1 is defined as $C\beta(P_1)$—$C\alpha(P_1)$—CH—OH.
[c] Dihedral #2 is defined as $C\beta(P_1)$—$C\alpha(P_1)$—CH—$C(P_1')$.
[d] Because of the rules of stereochemistry, the relative stereochemistry of the central hydroxy group of the HMC (norstatine) moiety is reversed, even though the absolute stereochemistry is the same as for all the other functional groups.
[e] Because of the symmetry of the diols, the values of dihedral #1 are ambiguous. For purposes of this table, it is sufficient to say that both occurrences of dihedral #1 are gauche in every case.
[f] Because of the symmetry of the diols, the values of dihedral #2 are ambiguous. For purposes of this table, it is sufficient to say that both occurrences of dihedral #2 are trans in every case.

that the *syn* conformation is unusual. This point has previously been made by Wlodawer and Erickson.[31] This is somewhat misleading, because in the bound conformation of every known HIV-PR inhibitor, the geometric relationship between the P_1 side chain and the central hydroxyl group is gauche (Table V). *Syn* refers to the relative stereochemistry of two adjacent stereocenters; it is a two-dimensional representation, and contains no information about the three-dimensional relationship between the stereocenters. So, for example, compounds **1** and **3** are *syn*, whereas compound **2** is *anti*. The data in Table V confirm that it is unusual to find compounds with the same absolute stereochemistry at the central hydroxyl group as compounds **1** and **3**. However, the critical issue is that the stereochemistry of the central hydroxyl group is linked to changes in the main-chain conformation on the prime side and the class of inhibitor, as explained previously. Changes to the ''core'' of the inhibitor—for example, from hydroxyethylamine (HEA) to statine to norstatine—will influence both the preferred backbone conformation and the preferred stereochemistry of the central hydroxyl group. The gauche configuration of the central OH group with respect to the preceding P_1 side chain *is required* by the HIV-PR active site (as seen in the crystal structures listed in Table V) to have optimal interaction between the OH and both catalytic aspartate side chains, as well as a strong hydrophobic interaction of the P_1 side chain with the S_1 pocket. In the gauche configuration, the hydroxyl oxygen is almost equidistant to the four Asp side-chain oxygens and is in the plan of these four oxygens. In spite of this stringent requirement, the configuration of the OH group is seen to invert (from *S* to *R* as we go down the list in the table from HE to HEA inhibitors), which is accomplished by changing the backbone configuration on the prime side as explained previously, as well as in our earlier studies.[13, 14] The first backbone carbon (or methylene

group) on the P′ side of the central hydroxyl can adopt either a gauche or trans configuration with respect to the P_1 side chain to place the *R*- or *S*-hydroxyl group in the middle of the two Asp side chains.

COMPARISON OF PROLINE-BASED INHIBITORS AND SUBSTRATES

As stated previously, the model of epi-KNI adopts the same backbone configuration as JG-365, a Phe–Pro-based inhibitor (Fig. 8). (Epi-KNI has an *R*-relative stereochemistry for the central hydroxyl group, which because of the rules of stereochemistry is the same absolute configuration as *S* JG-365.) the P_1' five-membered rings of the two inhibitors have a good overlap. These five-membered rings fill the S_1' site much better than substrates with proline at P_1', because the inhibitors have an extended backbone connecting the P_1 and P_1' groups. The backbone conformation of KNI-272 (Fig. 9) is clearly different, because of the alteration of absolute stereochemistry at the central hydroxyl. Furthermore, the P_1' ring of KNI-272 does not go as deeply into the pocket as epi-KNI or JG-365, but it is still better than substrate Pro in terms of filling the pocket and its ''edge-to-edge'' interaction with Pro-81′ of the enzyme. In all cases, the P_1' carbonyl is oriented similarly with respect to the five-membered ring. The T1 torsion (N2—C3—C4—C5) is always roughly −100° (substrate model = −116°, JG-365 X-ray = −89°, KNI X-ray = −98, epi-KNI model = −115°).

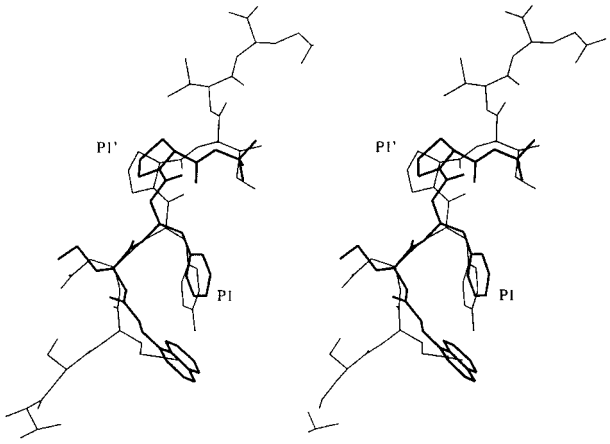


FIGURE 9. A comparison of the modeled substrate conformation (thin lines) with the bound X-ray conformation of KNI-272 (thick lines).

Conclusions

We summarize the most important points discussed in this work:

1. KNI-272 and related inhibitors are still quite flexible, and may adopt a wide range of conformations. The manifold of low-energy conformations available for binding includes cis amides. The preference for a trans amide conformation when bound to the active site of HIV-PR has nothing to do with an ''intrinsic'' preference for trans versus cis in these molecules.
2. KNI-272 is still somewhat strained in the bound state. Our estimates range from 2 to 3 kcal/mol just for the central norstatine–thioproline linkage. The mechanism of the strain is primarily the rotation of the carboxamide moiety, required to preserve the interaction with flap water. The origin of the strain is identical to that previously found[13,14] for the related molecule Saquinovir (**3**).
3. Quantitatively, the strain is somewhat lower for KNI-272 because the DIQ P_1' group in Saquinovir has been replaced by the simpler thioproline ring. Thus, the allophenylnorstatine–thioproline moiety in KNI-272 may be viewed as an ''improved P_1–P_1' scaffold'' with respect to strain energy.[32]
4. The comparison between KNI-272, epi-KNI-272, Saquinovir, and JG-365 reinforces and expands our understanding of the relationship between stereochemistry and P′ functionality. Additional structural information for a wide range of inhibitor classes will allow us to further refine this understanding.
5. The calculations and modeling provide a much more solid explanation for the 200-fold potency advantage of KNI-272 relative to its epi isomer. In brief, the strain energy of the bound state of epi-KNI-272 (whether in a cis or trans amide conformation) is much higher (approximately 3 kcal/mol) than for KNI-272 itself.
6. The calculations and modeling on our prototype compound **4** (Tables III and IV and Supplementary Table S2) also show why the bound X-ray conformation of KNI-272 has a

trans amide rather than cis. Basically, the carboxamide rotamer, which is required for interacting with the flap water, is much more stable in the trans amide conformation. It is *not* because the cis amide conformations are inherently much less stable than trans.

7. The bound conformation of substrates which contain proline at P_1' have been modeled. The preferred carboxamide rotamer for substrate closely matches that of all the inhibitor classes studied, with an N—C—C—C torsion angle of roughly $-100°$. In this sense, all these inhibitor classes are good substrate mimics. However, the placement of the proline ring of the inhibitors is somewhat different than that found in substrate, with the inhibitors positioned more deeply in the S_1' pocket.
8. Finally, our results make it clear that, by applying the rules of conformational analysis,[15] it should be possible, at least in principle, to design novel inhibitors that can bind to HIV-PR with reduced strain energy, and thereby obtain improved potency.

Acknowledgments

We thank Mike Hale and the rest of the HIV-PR chemistry team for their careful reading of this manuscript. We thank Dr. John Erickson for the coordinates of KNI-272 and for helpful discussions.

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32. Of course, it is important to remember that the drug design process involves many issues including toxicity, metabolism, biodistribution, and the like. Strain energy is only one of these issues, and cannot be used as the sole indicator of whether one compound is ''better'' than another.